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Investigation of Colloidal Graphite as a Matrix for Matrix-Assisted Laser Desorption/Ionisation Mass Spectrometry of Low Molecular Weight Analytes

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Abstract

The analysis of low molecular weight compounds by Matrix-Assisted Laser Desorption/Ionisation mass spectrometry is problematic due to the interference and suppression of analyte ionisation by the matrices typically employed – which are themselves low molecular weight compounds. The application of colloidal graphite is demonstrated here as an easy to use matrix that can promote the ionisation of a wide range of analytes including low molecular weight organic compounds, complex natural products and inorganic complexes. Analyte ionisation with colloidal graphite is compared with traditional organic matrices along with various other sources of graphite (e.g. graphite rods and charcoal pencils). Factors such as ease of application, spectra reproducibility, spot longevity, spot-to-spot reproducibility and spot homogeneity (through single spot imaging) are explored. For some analytes considerable matrix suppression effects are observed resulting in spectra completely devoid of matrix ions. We also report the observation of radical molecular ions [M$^{-\bullet}$] in the negative ion mode, particularly with some aromatic analytes.

Introduction

Matrix-assisted laser desorption/ionisation (MALDI) is a well-established and powerful ionisation technique for the mass spectrometric (MS) analysis of non-volatile, thermally labile analytes[1,2]. The presence of the matrix (usually a UV active acid) is a distinct obstacle in the application of MALDI to the analysis of low molecular weight (LMW) analytes because the resulting spectra will not only contain peaks corresponding to the analyte, but also to molecular ions and cluster ions of the matrix itself[3]. These signals are generally known as the matrix-background signals and can lead to confusion during the analysis of LMW analytes because they obscure analyte signals through an 'overcrowding effect'. In extreme cases, the matrix ionises preferentially over the analyte, so that only the various matrix ions are observed – termed the 'analyte suppression effect'[3]. In order to improve the utility of MALDI for the analysis of LMW analytes it is vital to try to significantly reduce, or totally eliminate, the matrix ions, leading instead to the highly desirable 'matrix suppression effect' (MSE).

The MSE has been extensively studied previously and the effects of certain key parameters such as laser intensity and analyte-to-matrix ratios have been established[4,5]. The analyte-to-matrix ratio has a significant effect on the balance of analyte suppression and matrix suppression observed. If it is optimum, total matrix suppression can be observed, however finding the optimum values is largely down to trial-and-error and would be impracticable in most laboratories.

Other methods have used additives to compete with the matrix for protons or the use of surfactants, for example cetrimonium bromide[6,7]. It is crucial that the additives do not behave as a matrix in themselves or that they do not produce significant signals in the spectra. Ideally any additives would react with excited matrix ions to prevent them from being detected. Another attempt to increase the MSE utilised a mixture of matrices: one acidic, and one basic[8]. Competition between the matrices for protons leads to the formation of only one peak for each matrix, and reduces the presence of other peaks due to matrix clustering. The combination of the two matrices also appears to reduce noise by lowering the threshold laser fluence needed for successful desorption. This reduction in background noise eases identification of analyte peaks in the low-mass region. The major restriction of the dual matrix method is that the analyte pK_a must lie outside the pK_as bracketed by the matrices in order to see successful matrix suppression. This leads to obvious difficulties for the analysis of previously unknown or newly synthesised analytes.

In 1987, Tanaka *et al.* reported the use of Ultra Fine Metal Powder (UFMP) as a matrix for MALDI[9,10]. The idea behind the application of UFMP was that the extremely small size of the metal particles would lead to super-quick heating of a sample upon laser irradiation and so induce desorption without fragmentation. This had some success but it was not until the UFMP was mixed with a glycerol solvent that it became useful for the successful desorption of thermally labile molecules. The UFMP/Glyercol mix led to efficient transfer of laser energy to the analyte *i.e.* the mixture behaved in the same way as we now see a matrix behave.

Studies have been performed using carbon nanotubes as a matrix for MALDI with results for analysis of small molecules showing a promising level of matrix suppression[11]. The carbon nanotubes are used slightly differently to traditional matrices, in that they provide multiple surfaces onto which crystallisation of the analyte takes place. This produces a more homogeneous sample spot easing sample preparation. The use of other carbon based matrices, such as Buckminsterfullerene and iron-cored carbon balls, have also recently been demonstrated to be effective matrices[12-14].

Several studies have suggested the use of graphite as an alternative matrix for MALDI because it can disperse energy to an analyte extremely effectively[15]. Graphite has been used in various forms; as a suspension, as a powder, or as a surface[16-20]. It is also a favourable choice for detection of LMW analytes due to the tendency of spectra to contain very few matrix peaks in the low-mass region.

Early attempts to use graphite as a matrix by Sunner *et al.* used liquid suspensions of graphite particles in glycerol[16]. This work focused mainly on peptides and proteins as analytes, but hinted at the potential for graphite for use with LMW compounds by noting the reduction in congestion in the low-mass region compared to traditional matrices. A study by Peng *et al.*, employed graphite suspensions in 2-propanol to perform Surface Assisted Laser Desorption/Ionisation from the surface of a Thin Layer Chromatography (TLC) plate[18]. This allowed for the direct analysis of the separated species without the previous problems involving diffusion of the compounds on the TLC plate after the matrix was applied and further demonstrated the ability of graphite as a matrix. Colloidal graphite has also been used to detect cerebrosides directly from rat brain tissue[15]. This method used either an aerosol spray or an airbrush to deposit the matrix directly onto the stainless steel plate containing the rat brain tissue slice. This allowed for 2D mass profiles for the lipids to be produced by MALDI imaging.

The first recorded use of pencil lead in Laser Desorption/Ionisation was by Chen *et al.* in 2001[21], but the practice of directly applying pencil lead to a plate for MALDI analysis was more recently established by Black *et al.* in 2006[22]. Pencil lead was found to be effective

matrix for the detection of a broad range of analytes and the scope of molecules analysed was extended to include a wide range of LMW analytes, polymers and organometallic species[23]. Sample preparation was shown to be very easy - the matrix can be deposited onto a plate, without the need for a solvent, simply by applying the pencil lead by hand *i.e.* by scribbling across the MALDI target plate.

In this study, we assess the utility of colloidal graphite as a matrix for a wide range of LMW analytes. Analyte and matrix suppression effects will also be explored along with technical issues such as spot-to-spot reproducibility, longevity and homogeneity (through single spot imaging).

Experimental

All analyses were performed on a 4700 Proteomics Analyser (Applied Biosystems, Warrington, United Kingdom). This is a reflectron Time-of-Flight/Time-of-Flight mass analyser, used in reflectron mode. All spectra were recorded at 1000 shots per spectrum (comprised of 8 sub-spectra, over a 5 s run). The laser used was a 200 Hz Nd/YAG at a wavelength of 355 nm. The laser beam diameter is 50 μm with pulse energy of 12 μJ and pulse length of less than 500 ps.

The pencils used were either Mars Lumograph grades 3B, 4B or 8B (Staedtler UK, Rhondda Cynon Taff, U.K.) or charcoal grades M, D or C (Derwent, Keswick, U.K.). The pencils were all sourced locally from 'high-street' stationers or artists' supply shops. The colloidal graphite was Edelgraphit Graphite Aerosol Spray (Graphite Trading Company, Birmingham, U.K.). The commercially available analytes and traditional matrices 2,5-dihydroxybenzoic acid (DHB) and sinapinic acid (SA) were all sourced from Sigma-Aldrich (Gillingham, U.K.). All solvents were HPLC gradient grade obtained from Fisher Scientific (Loughborough, U.K.)

All analytes were dissolved in an appropriate organic solvent (methanol, dichloromethane, chloroform or acetonitrile) to 1mg/mL and used without any further preparation or dilution. The traditional matrices 2,5-dihydroxybenzoic acid (DHB) and sinapinic acid

(SA) were dissolved in methanol to 10mg/mL. Colloidal graphite matrix was prepared by spraying approximately 1mL of the colloidal suspension from the aerosol spray can into a glass vial. This was allowed to evaporate and then re-suspended in 1mL of methanol/water (90:10). Sample spots were prepared by thoroughly mixing the analyte and matrix solutions in a 1:1 ratio. 1μL of this mixture was spotted on the MALDI target plate and allowed to dry.

Results and Discussion

Initial studies compared the ability of graphite and conventional matrices to ionise simple organic compounds, exemplified by the flavonoid hesperetin. A typical laser desorption ionisation (LDI) mass spectrum of hesperetin was performed as a control. The analyte solution (1 mg/mL in methanol) was spotted directly onto a MALDI target plate and analysed as with a standard MALDI experiment and the resulting spectrum is shown in Figure 1. This spectrum clearly demonstrates that hesperetin can be successfully ionised by LDI (*i.e.* without a matrix) but the signal intensity is very low (at just 182 counts) and the spectrum is very noisy. An attempt to perform negative ion LDI on hesperetin resulted in no identifiable analyte peaks in the spectra (data not shown).

The MALDI-MS analysis of hesperetin with a range of matrices is illustrated in Figure 2. Using 2,5-dihydroxybenzoic acid (DHB) as matrix (Figure 2a) results in intense ions due to the sodiated and potassiated matrix (*m/z* 177 and 193 respectively) along with a range of matrix cluster ions and impurities in the region from *m/z* 300 to 800. There is a small peak at *m/z* 303 for the protonated analyte, but we cannot unambiguously confirm the identity of this peak due to its low intensity. The performance of sinapinic acid (SA) (Figure 2b) is considerably worse than DHB as this shows almost total analyte suppression by the matrix with a range of intense matrix molecular ions and clusters from *m/z* 200 to 650.

The poor performance of these traditional matrices for the analysis of LMW analytes stimulated the initial studies by this group to reproduce the work using pencil matrices by Langley *et al.*[22,23]. These studies identified some problems with the utilisation of direct pencil lead as a matrix for MALDI. The practice of applying the pencil directly to the plate

by rubbing or scribbling was found to cause considerable scratching of the plates. This effect occurred for all pencils used, but was most apparent with the use of harder grade pencils, (4B and upwards) where significant scratching was observed. Scratching of the plates led to them being unusable, because any liquid analyte or matrix added to the plate tended to flow along the scratches in the plate, leading to inhomogeneous sample spots and cross-contamination. Whilst the application procedure was relatively easy, it was largely subjective due to variables such as the sharpness of the pencil, and the pressure with which it is applied to the surface of the plate. Decreased reproducibility was also an issue due to the inability of the user to clearly see the matrix as it was deposited leading to varying amounts of matrix being added to the plate for each analysis. These problems with using pencil-based matrices initiated the search for improved methods of graphite deposition that would reduce or eliminate the possibility of damaging the plates and yield a more consistent matrix surface.

In 2007, Cha and Yeung reported the use of colloidal graphite as the matrix in a MALDI imaging experiment to detect cerebrosides directly from rat brain tissue[15]. Investigations by this group led to the discovery of a commercially available aerosol spray containing a colloidal suspension of graphite in alcohol (used as an industrial lubricant) and it was thought that this was maybe a novel method of application of graphite to a MALDI sample plate. Initial experiments were performed by spraying the aerosol directly onto the MALDI target plate, but this led to similar issues with reproducibility to using pencils – i.e. how long to spray, how far to hold the spray can away from the plate and how much pressure to apply to the aerosol nozzle. However, analysis of a spot covered with a fine film from the aerosol produced a spectrum showing intense carbon cluster peaks with no other contaminants (data not shown).

An alternative method for the application of the colloidal graphite spray was to spray a quantity of the aerosol into a vial and to re-suspend the graphite with methanol/water (90:10). This suspension could then be deposited by pipette onto the MALDI target plate in much the same way as traditional matrices. This gives the operator much more control over the matrix deposition and enables confinement of the matrix to a single spot rather

than covering a whole area in one go by spraying the aerosol. Unlike pipette deposition of established MALDI matrices however, the concentration of graphite in the aerosol and hence the suspension is unknown, thus the quantity of graphite deposited onto the MALDI target plate is also unknown.

The resulting spectra of hesperetin using 4B pencil lead and the pipetted graphite as matrices are also shown in Figure 2. With 4B pencil (Figure 2c) there are very intense intact molecular species for hesperetin (m/z 303 [M+H]$^+$, 325 [M+Na]$^+$ and 341 [M+K]$^+$) but also some intense background ions due to pencil lead additives (glide agents, waxes, clays). The intensity of the hesperetin ions here is approximately an order of magnitude greater than that seen with DHB. With pipetted colloidal graphite (Figure 2d), the same molecular species are observed, but with considerable matrix suppression and no background ions resulting from matrix impurities. The intensity here is slightly lower than that observed with pencil lead, but the quality of the spectrum is far superior. There are a few low-mass peaks due to carbon clusters and carbon oxide clusters, but these are of very low intensity (less than 10% of the intensity of the hesperetin [M+Na]$^+$).

The analysis was repeated in negative ion mode (see Figure 3) using the same sample preparations to those shown in Figure 2. MALDI-MS spectra of hesperetin using the matrices DHB and SA are shown in Figures 3a and 3b respectively. They show almost identical results and so can be discussed jointly. In both cases, the most abundant ion is the [M-H]$^-$ of the matrix at m/z 153 and m/z 223 respectively. There is also a high intensity peak for the [M-H]$^-$ of the analyte at m/z 301. Both spectra also show peaks for matrix cluster ions. These spectra are of reasonable quality, but it should be noted that the signal intensity is very low (at approximately 150 counts) and even though the relative intensities of the analyte ions are quite high, they are still not the most abundant ions in the spectra.

The negative ion analysis of hesperetin using pencil lead and colloidal graphite are shown in Figures 3c and 3d respectively. They are also quite similar and can be discussed together. They both show intense [M-H]$^-$ analyte ions at m/z 301 and interestingly also show very intense M$^{\bullet-}$ ions at m/z 302. In the case of colloidal graphite the radical ion is almost as

intense as the deprotonated molecular ion. The mechanism of formation of the radical ion is unknown but we believe it is the result of electron transfer from the graphite to the aromatic system of the analyte. The intensity of the analyte ions in these spectra are almost 2 orders of magnitude higher than with DHB and SA. Another interesting feature of these two spectra is the presence of fragment ions. These occur no matter what laser power is used and so must be a feature of the ionisation and in fact they seem to be due to fragmentation of the radical ion in similar ways to those expected in electron ionisation. In both spectra, peaks are observed for the loss of a methyl radical ($[M-CH_3]^-$ at m/z 287) and for the loss of a hydroxide radical ($[M-OH]^-$ at m/z 285). There are also several additional fragment ions at lower m/z. Both spectra also contain peaks due to carbon clusters (of the type C_n^-) with the intensity being much lower with colloidal graphite.

To understand the performance of colloidal graphite as a matrix, reproducibility studies were undertaken for DHB, 4B pencil lead and pipette-deposited colloidal graphite suspension, with hesperetin as the analyte. The study considered both the reproducibility of the most abundant molecular ion, with regard to multiple spectra within a single spot (intra-spot analysis) and between different spots (inter-spot analysis) of the same concentration. Spot longevity was also studied to determine how well the colloidal graphite behaved as a matrix over continuous repeated analyses. For these experiments, the instrument was set to a fixed laser power, and the same number of laser shots and sub spectra were used for each acquisition. The most abundant molecular ion was $[M+H]^+$ for DHB and $[M+Na]^+$ for the 4B pencil and colloidal graphite. As the concentration of hesperetin decreases, the general trend for all matrices is a decrease in the signal intensity of analyte molecular ions – *i.e.* as expected, the analyte is being increasingly suppressed by the matrix at lower concentrations with all matrices.

DHB generally shows low intra-spot reproducibility with intensity variation being much higher than the two carbon matrices tested. This is most likely due to the uneven way in which DHB crystallises when it is spotted - large crystals of analyte and matrix can form in some areas, with smaller crystals in others (see later). When using DHB, there is a tendency for both the matrix and analyte to diffuse to the edges of the spot, leading to high

analyte/matrix concentrations at the edges and low concentrations in the centre. Both these factors lead to the formation of inhomogeneous sample spots and low intra-spot reproducibility. The 4B matrix shows a slightly higher level of reproducibility than DHB, probably due to the grain size of the carbon particles present on the spot, which when compared to crystals of DHB are smaller and more consistent in size, leading to more consistent ionisation over the spot surface.

The pipetted graphite suspension showed a similar level of reproducibility to the 4B matrix but produced the highest signal intensities. This high performance is probably due to the method of application of the matrix to the plate - the colloidal graphite is applied to the plate by pipetting a fine suspension, producing a very thin and homogeneous layer of matrix over the whole spot (see later).

When looking at the absolute intensity values, spot-to-spot reproducibility for DHB appears to be very poor. The main peaks in the spectrum show considerable variations in relative intensities and the presence of additional peaks that together, further confuse the spectrum. As with intra-spot reproducibility, the poor reproducibility between spots is most likely a result of the inherent inhomogeneity of the spots as described above. Using the "dried-droplet" method of making up the DHB spots leads to a variation in spot size and layer thickness despite the same amount of matrix and analyte being added to each spot. Spot-to-spot reproducibility is notably better for 4B pencil lead. The improved performance over that of DHB is most likely due to the more consistent method of spot preparation for the 4B pencil. The application of a thin film of carbon to the spot is achieved by simply drawing directly onto the plate and therefore covering the whole surface of the spot fairly evenly. The problems associated with uneven crystallisation seen with DHB are largely avoided. The graphite suspension shows excellent of reproducibility for the main analyte molecular peaks with a slightly improved performance to that of 4B pencil lead. As noted during the intra-spot reproducibility analysis, this is made more significant when considering that the colloidal graphite produced the highest analyte intensity values of the matrices tested.

The issue of 'spot longevity' is clearly important when considering the utility of a novel MALDI matrix. To test the ability of colloidal graphite as a matrix, analyses were performed by continuous analysis of the same sample spot over an extended time period (see Figure 4) and compared to the traditional matrix, DHB, and the established carbon based matrix, 4B pencil lead. DHB produces the lowest intensity maximum for the $[M+Na]^+$ peak – which is probably as expected as DHB produces relatively small sodiated peaks when compared to carbon matrices. There are very high variations in intensity values illustrating the low level of spot homogeneity, with the highest intensity being observed after about one hour of continuous analysis (approximately 500 analyses) - see Figure 4a. Analysis of the $[M+H]^+$ peak from the same datasets shows that initially it is more intense than the $[M+Na]^+$ peak, but after about ten minutes the intensity has dropped to being just above the baseline (data not shown), whereas the $[M+Na]^+$ peak is continuously observable over the duration of the experiment.

The $[M+Na]^+$ peak shows extensive longevity for both of the carbon matrices. The number of runs before the signal intensity become unacceptable is well over a thousand and indeed, acceptable values are still being produced well after one hour of continuous analysis for the graphite suspension and for almost three hours for 4B pencil lead. Whilst 4B pencil showed more impressive longevity than the graphite suspension, the variation in intensity is clearly greater and the spectral quality clearly lower. This is due to the high level of spot homogeneity for the graphite suspension, producing more consistent spectra over time, and that colloidal graphite is a more pure form of carbon 'simplifying' the ionisation process when compared to pencil lead, which contains unknown additives and impurities.

Similar results are observed in negative ion mode, except that DHB is (as expected) even more erratic and that the most intense ions for the carbon matrices are the radical anions $(M^{\bullet-})$ rather than $[M-H]^-$ for DHB. The 4B pencil again shows greater longevity than colloidal graphite and the spectra are much cleaner than in positive ion mode, probably as a result of many of the impurities and additives in pencil lead not being ionisable in negative ion mode.

Due to the issues of glide agents and other additives present in the pencil lead, blank spectra were recorded in both positive and negative ion modes (see Figure 5) and compared to colloidal graphite in order to determine purity differences. The LDI spectra for 4B pencil lead in positive and negative ion modes are shown in Figures 5a and 5b respectively and it is immediately obvious that there are a lot of peaks that are not due to carbon. The low-mass end is dominated by the expected carbon clusters (C_n^+ or C_n^-), but there are a number of others peaks attributed to C16:0 and C18:0 fatty acid glide agents present in the pencil. These could have the undesirable effect of masking any analyte peaks in this region in much the same way as with the traditional matrices. Colloidal graphite shows very similar LDI spectra for the carbon clusters. In positive mode (figure 5c) these extend to about C_{25}^+ (*m/z* 300) and in negative mode (data not shown) they extend to about C_{12}^+ (*m/z* 144) and then drop away sharply. The spectra are completely devoid of any other ions.

So far, all the analyses have been performed using hesperetin as the analyte and it is clear that colloidal graphite is an excellent matrix for this specific analyte. In order to test the utility of the colloidal graphite as a matrix, a range of other LMW analytes both 'commercially available standards' and synthetic products were also studied (see Figures 6 and 7 for some selected examples and supplementary information). The spectra presented are fully representative and have been chosen to cover a wide range of chemical functionalities.

Figure 6 shows the positive ion graphite MALDI-MS analyses of several commercial samples. Figure 6a is of the cyclic depsipeptide valinomycin (Mw 1110 Da) and shows 2 analyte peaks: the [M+Na]$^+$ at *m/z* 1133 and the [M+K]$^+$ at *m/z* 1149. There are also peaks due to Na$^+$ and K$^+$ at low-mass. There are no other signals present – this spectrum is an excellent example of the total MSE (a hugely desirable result in MALDI-MS) and is quite typical of the positive ion spectra obtained with colloidal graphite as a matrix. Figure 6b is of the aminoglycoside kanamycin (Mw 484 Da) also shows total MSE and is dominated by peaks due to [M+Na]$^+$ (*m/z* 507) and [M+K]$^+$ (*m/z* 523). There are other peaks present in this spectrum; these are probably due to impurities as this sample was only 95% pure. Figure 6c is of the polyketide antibiotic oleandomycin (Mw 687 Da) is totally dominated

by [M+Na]+ and [M+K]+ at *m/z* 710 and 726 respectively. Again there are a few other peaks in this spectrum, which are most likely due to impurities in the sample. The ion at *m/z* 158 is almost certainly due to the [M+H]+ of the desosamine sugar, which is a commonly observed fragment ion in the MS analysis of this analyte[24].

Finally to test colloidal graphite as a matrix for the analysis of a mixture, an approximate 1:1:1 mixture of the quercetin glycosides (rutin, Mw 610 Da and isoquercetin, Mw 464 Da) with the quercetin aglycone (Mw 302 Da) was also analysed (Figure 6d). All three analytes produce intense peaks for their [M+Na]+ and [M+K]+ molecular adduct ions. Quercetin also has a low intensity [M+H]+ peak. There are a few unidentified signals in the *m/z* 280-350 range. What is clear from this spectrum is that total MSE has occurred again along with an apparent lack of any significant mass discrimination in the ionisation efficiency of these three species. This could be due to them having a similarly high cation affinity.

In order to test the versatility of colloidal graphite as a matrix, a representative selection of synthetic compounds from the School of Chemistry was analysed with the results presented in Figure 7. All samples had previously been analysed by other MS methods (EI and/or ESI) and also by NMR to test that the correct structures were as proposed. Figure 7a is the positive ion mode analysis of an amine ligand demonstrating total MSE with very intense ions due to [M+Na]+ (*m/z* 322) and [M+K]+ (*m/z* 338) with a less intense ion for [M+H]+ (*m/z* 300). Figure 7b is the positive ion mode spectrum of a cobalt porphyrin complex and shows an unexpected highly abundant ion due to the radical cation − suggesting that oxidation is the dominant ionisation route. There is also a surprising water adduct ion of the radical at *m/z* 713. There are some low intensity carbon cluster (matrix) ions in the *m/z* 100-250 range. Figure 7c is the positive ion mode analysis of a tridentate Rh catalyst showing a similar result with the radical cation dominating the spectrum. The ESI analysis of this analyte is very complex and is dominated by ligand exchange and fragmentation. There are also some lower intensity signals here due to fragment ions. Figure 7d is the negative ion mode spectrum of an aromatic diacid. The most abundant ions are due to deprotonation (*m/z* 215) and loss of carbon dioxide (*m/z* 171). There is also a small analyte cluster ion with sodium at *m/z* 453. This is very similar to the spectrum observed with

negative ESI analysis. Figure 7e is the negative ion mode spectrum of an organotrifluoroborate. These species are quite difficult to analyse by MS, but here an excellent mass spectrum is observed with a highly dominant molecular ion (m/z 201) and dimer with potassium (m/z 441). Figures 7d and 7e show typical carbon cluster matrix peaks in the m/z 100 – 150 range. So far a total MSE has not been observed in negative ion mode. Of all the analytes studied here, only a few failed to produce recognisable high-intensity analyte ions (adduct ions or radical cations in positive ion mode, deprotonated ions in negative ions) with some inorganic species producing positive ions through loss of an anionic counter ion (Cl⁻, Br⁻, triflate etc.). The remaining analytes, were most monodentate inorganic species that tended to fragment extensively. In most cases intact molecular ions were present, but only at highly reduced intensities relative to other peaks in the spectrum. It must be noted that these types of analytes are notoriously difficult to analyse by MS and their purities were also unknown.

Another way of studying matrix performance is by single spot MALDI imaging. In this part of the study, single spots of two LMW analytes representing two structural extremes (hesperetin being fully unsaturated and 1,2-di-palmitoyl-*rac*-glycerol being completely saturated) were analysed by MALDI imaging with the range of matrices. This was to allow a comparison of the performance of each matrix in terms of homogeneity. It should be noted, that although the 1,2-di-palmitoyl-*rac*-glycerol was supplied as 99% pure, spectra produced peaks at m/z = 273 and 295 corresponding to protonated and sodiated palmitic acid respectively. This leads to the conclusion that the sample was in fact a mixture of the 1,2-di-palmitoyl-*rac*-glycerol and palmitic acid and analysis by other techniques confirmed that.

The DHB image shows poor ionisation with hesperetin (see Figure 8). The [M+H]⁺ image shows low spot homogeneity, with intense peaks produced over small areas of the spot ("sweet spots") and large areas producing zero intensity ("dead spots"). This corresponds with the large levels of variation in intensity seen during reproducibility and longevity experiments. A result of the low spot homogeneity is the need to carry out time consuming searches for these "sweet spots" in order to produce high quality spectra. The spot

inhomogeneity also induces a level of subjectivity into spot analysis because the user themselves has to decide on when a "sweet spot" has been found. More crucially, this prevents automated analysis. There is a noticeable ring of higher analyte concentration at the edges of the spot, with very low concentrations at the centre. This supports previous observations that diffusion to the edges of the spot of both matrix and analyte is taking place, and further demonstrates the poor performance of DHB as a matrix for MALDI analyse of LMW analytes. As expected, the $[M+Na]^+$ and $[M+K]^+$ images show almost no ions, the dominant ionisation process being protonation of the analyte.

When spotted with 1,2-di-palmitoyl-*rac*-glycerol however, extremely low intensity peaks are produced for the $[M+H]^+$ ion, and these images are not shown. This suggests that DHB is undergoing preferential protonation of another species on the spot, and the high intensity of the protonated palmitic acid peak suggests further that this is the case. A very distinct ring of high intensity is observed for $[M+Na]^+$, with analyte molecular ions being produced almost exclusively from the edges of the spot. This again highlights problems involving diffusion of analyte and matrix to the spot edges when using DHB. This ring effect was also seen for the palmitic acid component of the mixture, although intense spectra were produced from much larger areas of the spot. This was hypothesised to be largely due to self-ionisation, however a spectrum of the analyte spotted with no matrix showed very low intensity, suggesting the matrix was in fact assisting the desorption/ionisation of the palmitic acid. A possible explanation is reached when the relative pK_as of palmitic acid and DHB are considered (pK_a palmitic acid = 9 and pK_a DHB = 3). The DHB readily protonates the palmitic acid, causing large amounts of preformed protonated species to be present on the plate, and hence seen in the resulting spectrum. This simple protonation is acid/base chemistry, and is removed from the MALDI mechanism. This may also explain the low intensity of the $[M+H]^+$ peak for 1,2-di-palmitoyl-*rac*-glycerol, since all available protons are used to ionise the palmitic acid component. The sodiated palmitic acid peak shows low intensity. Again this is due to the dominance of protonation of the analyte that occurs with DHB. A faint ring is still observed, further indicating that radial diffusion is taking place to the edges of the plate. In order to obtain a satisfactory spectrum the sample had to be irradiated with higher laser fluence than was required to induce ionisation for

colloidal graphite, resulting in degradation of the sample spot, removing large amounts of material, making the spot largely unsuitable for further use.

For both hesperetin and 1,2-di-palmitoyl-*rac*-glycerol the 4B matrix produced a clear ring of higher intensity at the spot edges (see Figures 8 and 9). This is probably a result of migration of the analyte to the spot edges due to repulsive interactions between the polar solvent and the hydrophobic fatty acids/wax esters that are added to the pencil lead during manufacture. Also, adsorption of analyte molecules to the raised surface at the edges of the spot could lead to higher analyte concentrations, again producing a ring of high intensity. Although fairly low intensity, the spectra from other areas of the spot are relatively consistent for the cationised molecular analyte ions, showing an improvement on the spot homogeneity seen for DHB. The $[M+H]^+$ peak for both hesperetin and palmitic acid is very low intensity due to the 4B matrix acting as a less abundant proton source than DHB. Also, the sodiated palmitic acid peak shows significantly higher intensity than that of 1,2-di-palmitoyl-*rac*-glycerol. This suggests a preference for cationation with palmitic acid. Despite improved performance over DHB, in order to obtain high intensity spectra a search for "sweet spots" must still be undertaken.

The analysis of hesperetin with the colloidal graphite suspension produced sample spots of extremely high homogeneity, with high quality spectra obtained throughout the spot for the three major analyte molecular ions. As described in previous experiments, this is most likely due to the method of application of the matrix to the plate surface - the deposition by pipette of a fine suspension leads to an extremely uniform spot, and an even layer of carbon added to each spot. This means the search for a "sweet spot" is almost entirely removed, as are any subjective decisions over when a "sweet spot" has been found; and unlike when using DHB and the 4B pencil, automated analysis of a spot is now possible. This would allow many samples to be tested extremely quickly and efficiently, using unattended batch analysis methods. Also, this method of deposition applies a very consistent amount of carbon to each spot. Indeed, it would be possible to prepare suspensions of precisely known concentration, meaning the exact amount of carbon added to each spot can be determined, and hence controlled. This presents clear advantages over

the application of the 4B pencil matrix, where previously described variable factors as well as the inherent invisibility of the applied carbon can lead to large variations in the absolute amount of carbon added to each spot. The major $[M+Na]^+$ peak is of significantly higher intensity than the major peak seen in the DHB spectrum ($[M+H]^+$), indicating that more efficient ionisation is also taking place.

When spotted with 1,2-di-palmitoyl-*rac*-glycerol, an equally high level of spot homogeneity is seen, despite the overall intensity for the molecular ion being lower than that of DHB. As with the 4B matrix, it appears that cationisation with sodium is preferred for the palmitic acid component of the mixture over the molecular ion, leading to the low intensity of the sodiated 1,2-di-palmitoyl-*rac*-glycerol peak. The protonated palmitic acid peak shows almost zero intensity, due to the colloidal graphite acting as a source of metal ions, and a less abundant source of protons. However, the extremely high homogeneity and peak intensity for the sodiated palmitic acid peak does further illustrate the improved performance of the colloidal graphite matrix over that of DHB.

Conclusions

The carbon matrices tested showed significant improvements on the performance of the established organic matrices DHB and SA for the analyses of LMW analytes. DHB was shown to produce spots of poor homogeneity, leading to poor reproducibility in both the intra-spot and inter-spot analyses. Significant degradation of the spot over time was demonstrated by the poor spot longevity, and the presence of several large matrix peaks in the low-mass range results in extensive analyte suppression. This makes DHB a poor choice of matrix for analysis of LMW analytes.

The 4B pencil lead shows variable performance. Despite the observation of consistently intense molecular ions in the spectra and excellent spot longevity, the application method leads to poor spot homogeneity and reproducibility, as well as subjectivity issues that prevent automated analysis. In addition to this, the application of graphite by scribbling the pencil on the sample plate causes damage to the plate and plate cleaning issues. The

presence of glide agents highly complicates the low mass range, particularly at low analyte concentration or for the analysis of analytes with low ionisabilities, again meaning analysis of LMW analytes is difficult.

The colloidal graphite matrix was investigated using two application methods – spraying directly onto the sample plate, and by pipetting. Both methods produced high intensity analyte molecular ions, almost total matrix suppression and extensive spot longevity. The graphite spray showed several disadvantages however. Although appearing to exhibit high levels of intra- and inter-spot reproducibility from the same application of matrix, suggesting high spot homogeneity, there is a large variation seen with each separate matrix application. This would presumably lead to poor reproducibility between plates prepared at different times, and hence make comparison of results difficult. The colloidal suspension deposits an extremely thin and even film of carbon onto the spot, leading to high spot homogeneity, and hence excellent reproducibility. Application methods are extremely consistent, potentially with control over the exact amount of carbon added to each spot. Again, further work would indicate if this were practical. Colloidal graphite showed excellent quality spectra for a range of LMW analytes. Further work is required to establish whether colloidal graphite is a good all round matrix for all LMW analytes, but this study clearly demonstrates it has enormous potential.

References:

[1] M. Karas, D. Bachmann, U. Bahr, F. Hillenkamp. Matrix-assisted ultraviolet laser desorption of non-volatile compounds. *Int. J. Mass Spectrom. Ion Proc.* 1987, *78*, 53.

[2] M.Karas, U. Bahr, U. Giessmann. Matrix-assisted laser desorption ionization mass Spectrometry. *Mass Spectrom. Rev.* 1991, *10,* 335.

[3] R. Knochenmuss, R. Zenobi. MALDI Ionization: The role of in-plume processes. *Chem. Rev.* **2003**, *103*, 441.

[4] G. McCombie, R. Knochenmuss. Small-molecule MALDI using the matrix suppression effect to reduce or eliminate matrix background interferences. *Anal. Chem*. **2004**, *76*, 4990.

[5] T. W. D. Chan, A. W. Colburn, P. J. Derrick. Matrix-assisted UV laser desorption. Suppression of the matrix peaks. *Org. Mass. Spectrom*. **1991**, *26*, 342.

[6] S. Vaidyanathan, S. Gaskell, R. Goodacre. Matrix-suppressed laser desorption/ ionisation mass spectrometry and its suitability for metabolome analyses. *Rapid Commun. Mass Spectrom.* **2006**, *20*, 1192.

[7] Z. Guo, Q. Zhang, H. Zou, B. Guo, J. Ni. A method for the analysis of low-mass molecules by MALDI-TOF mass spectrometry. *Anal. Chem*. **2002**, *74*, 1637.

[8] Z. Guo, L. He. A binary matrix for background suppression in MALDI-MS of small molecules. *Anal. Bioanal. Chem.* **2007**, *387*, 1939.

[9] K. Tanaka, Y. Ido, S. Akita, Y. Yoshida, T. Yoshida. *Proceedings of the Second Japan-China Joint Symposium on Mass Spectrometry*, 1987, 185.

[10] K. Tanaka, H. Waki, Y. Ido, S. Akita, Y. Yoshida, T. Yoshida. Protein and polymer analyses up to *m/z* 100 000 by laser ionization time-of-flight mass spectrometry. *Rapid Commun. Mass Spectrom*. **1988**, *2*, 151.

[11] S. Y. Xu, Y. F. Li, H. F. Zou, J. S. Qiu, Z. Guo, B. C. Guo. Carbon nanotubes as assisted matrix for laser desorption/ionization time-of-flight mass spectrometry. *Anal. Chem*. **2003**, *75*, 6191.

[12] L. Michalak, K. J. Fisher, D. S. Alderdice, D. R. Jardine, G. D. Willett. C(60)-assisted laser desorption-ionization mass spectrometry. *Org. Mass. Spectrom*. **1994**, *9*, 512.

[13] J. P. Huang, C. H. Yuan, J. Shiea, Y. C. Chen. Rapid screening for diuretic doping agents in urine by C(60)-assisted laser-desorption ionization time-of-flight mass spectrometry. *J. Anal. Toxicol.* **1999**, *23*, 337.

[14] H. S. Wen, L. F. Tsai, C. W. Liu, Y. C. Tyan, S. L. Shy, B. T. Dai, P. C. Liao, C. Y. Wang. Effects of iron-cored carbon balls as matrix on matrix-assisted laser desorption ionization-time of flight mass spectrometer analysis. *Jpn. J. Appl. Phys.* **2007**, *46*, 6415.

[15] S. Cha, E. S. Yeung. Colloidal graphite-assisted laser desorption/ionization mass spectrometry and MS^n of small molecules. 1. Imaging of cerebrosides directly from rat brain tissue. *Anal. Chem.* **2007**, *79*, 2373.

[16] J. Sunner, E. Dratz, Y. C. Chen. Graphite surface-assisted laser desorption/ionization time-of-flight mass spectrometry of peptides and proteins from liquid solutions. *Anal. Chem.* **1995**, *67*, 4335.

[17] M. J. Dale, R. Knochenmuss, R. Zenobi. Graphite/liquid mixed matrices for laser desorption/ionization mass spectrometry. *Anal. Chem.* **1996,** *68*, 3321.

[18] S. Peng, M. Elder, N. Ahlmann, N. Hoffman, J. Franzke. A new interface to couple thin-layer chromatography with laser desorption/atmospheric pressure chemical ionization mass spectrometry for plate scanning. *Rapid Commun. Mass Spectrom.* **2005**, *19*, 2789.

[19] S. Zumbuhl, R. Knochenmuss, S. Wulfert, F. Dubois, M. J. Dale, R. Zenobi. A graphite-assisted laser desorption/ionization study of light-induced aging in triterpene dammar and mastic varnishes. *Anal. Chem.* **1998**, *70*, 707.

[20] J. Kim, K. Paek, W. Kang. Visible surface-assisted laser desorption/ionization mass spectrometry of small macromolecules deposited on the graphite plate. *Bull. Korean Chem. Soc.* **2002,** *23*, 315.

[21] Y. C. Chen, J. Y. Wu. Analysis of small organics on planar silica surfaces using surface-assisted laser desorption/ionization mass spectrometry. *Rapid Commun. Mass Spectrom.* **2001**, *15*, 1899.

[22] C. Black, C. Poile, J. Langley, J. Herniman. The use of pencil lead as a matrix and calibrant for matrix-assisted laser desorption/ionisation. *Rapid Commun. Mass Spectrom.* **2006**, *20*, 1053.

[23] G. J. Langley, J. M. Herniman, M. S. Townell. 2B or not 2B, that is the question: further investigations into the use of pencil as a matrix for matrix-assisted laser desorption/ionisation. *Rapid Commun. Mass Spectrom.* **2007**, *21*, 180.

[24] M. C. Crowe, J. S. Brodbelt, B. J. Goolsby, P. Hergenrother. Characterization of erythromycin analogs by collisional activated dissociation and infrared multiphoton dissociation in a quadrupole ion trap. *J. Am. Soc. Mass Spectrom.* **2002**, *13*, 630.

Legends to figures:

Figure 1: Positive ion LDI-MS of hesperetin (Mw = 302 Da) serving as a control experiment. No recognisable signals for hesperetin are observed in the negative ion LDI analysis.

Figure 2: Positive ion MALDI-MS spectra of hesperetin (Mw = 302 Da) with a range of different matrices. Spectrum (a) is DHB, (b) is sinapinic acid, (c) is 4B pencil lead and (d) is pipetted colloidal-graphite. Analyte suppression is clearly observed in spectra (a) and (b) and matrix suppression in spectra (c) and (d). Ions due to the matrix (X) are indicated in spectra (a) and (b).

Figure 3: Negative ion MALDI-MS spectra of hesperetin (Mw = 302 Da) with a range of different matrices. Spectrum (a) is DHB, (b) is sinapinic acid, (c) is 4B pencil lead and (d) is pipetted colloidal-graphite. Ions due to the matrix (X) are indicated in spectra (a) and (b). The insert in spectrum (d) shows an expansion of the molecular ion region clearly showing the generation of the radical ion and fragment ions.

Figure 4: Results of the longevity experiment to test the performance of the colloidal graphite matrix against the established matrices DHB and 4B pencil led. In all cases the analyte is hesperetin at 1 mg/mL. Graphs (a), (d) and (g) show a plot of signal:noise ratio *vs.* spectrum number. Five hundred spectra equate to approximately 1 hour of continuous ablation. Graph (a) is for DHB, (d) is for pencil lead and (g) is for pipetted colloidal graphite. Spectra (b) and (c) are spectra 1 and 500 from DHB. Spectra (e) and (f) are spectra 1 and 500 from 4B pencil lead. Spectra (h) and (i) are spectra 1 and 500 from colloidal graphite.

Figure 5: LDI mass spectra of various graphite sources (performed as a baseline analysis). Spectrum (a) and (b) are of 4B pencil lead in positive and negative ion modes respectively. Spectrum (c) is of colloidal graphite in the positive ion mode.

Figure 6: Positive ion MALDI mass spectra of various LMW standards using pipetted colloidal graphite as the matrix. Spectrum (a) is the cyclic depsipeptide valinomyicn, (b) is the aminoglycoside kanamyicn, (c) is the polyketide oleandomycin and (d) is a mixture of the flavonol glycosides rutin (R, Mw 610) and isoquercetrin (I, Mw 464) with the quercetin aglycone (Q, Mw 302).

Figure 7: MALDI mass spectra of various LMW synthetic compounds using pipetted colloidal graphite as the matrix. Spectrum (a) is of an amine ligand (Mw 299 Da), (b) is of a cobalt porphyrin complex (Mw 694 Da), (c) is of a Rh catalyst (Mw 1101 Da), (d) is of an aromatic diacid (Mw 216) and (e) is of a organotrifluoroborate (Mw 201 Da). Spectra (a), (b) and (c) are positive ion mode and (d) and (e) are negative ion mode.

Figure 8: MALDI 'single spot' images comparing various molecular ion intensities of Hesperetin (1mg/mL) for 3 matrices: DHB, 4B pencil lead and pipetted colloidal graphite. The ions studied were m/z 303 = [M+H]$^+$, m/z 325 = [M+Na]$^+$ and m/z 341 = [M+K]$^+$. The intensity scale is an arbitrary relative scale, ranging from red (highest intensity) to violet (lowest).

Figure 9: MALDI 'single spot' images comparing molecular ion intensities of a mixture of 1,2-dipalmitoyl-*rac*-glycerol and palmitic acid mixture (1mg/mL) for 3 matrices: DHB, 4B pencil lead and pipetted colloidal graphite. The ions studied were m/z 591 = [M+Na]$^+$ of 1,2-dipalmitoyl-*rac*-glycerol, m/z 273 = [M+H]$^+$ of palmitic acid and m/z 295 = [M+Na]$^+$ of palmitic acid. The intensity scale is an arbitrary relative scale, ranging from red (highest intensity) to violet (lowest).